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Plasma Levels of MASP-1 and MASP-2 are Elevated in Type 1 Diabetes and Correlate with Glycaemic Control

Short title: MASPs in Diabetes

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Abbreviations: ANOVA = analysis of variance; BMI = body mass index; ELISA = enzyme-linked immunosorbent assay; HbA1c = glycated haemoglobin type A1c; IQR = interquartile range; MASP = mannan-binding lectin-associated serine protease; MBL = mannan-binding lectin; T1DM = type 1 diabetes mellitus; T2DM = type 2 diabetes mellitus

Summary

There is increasing evidence that the complement system plays an important role in diabetes and the development of diabetic vascular complications. In particular, mannan-binding lectin (MBL) levels are elevated in diabetes patients, and diabetes patients with diabetic nephropathy have higher MBL levels than diabetes patients with normal renal function. The MBL-associated serine proteases (MASPs) MASP-1, MASP-2, and MASP-3, and MBL-associated protein MAp44 have not yet been studied in diabetes patients. We therefore measured plasma levels of MASP-1, MASP-2, MASP-3, and MAp44 in 30 children with type 1 diabetes mellitus (T1DM) and 17 matched control subjects, and in 45 adults with T1DM and 31 matched control subjects. MASP-1 and MASP-2 levels were significantly higher in children and adults with T1DM than in their respective control groups, whereas MASP-3 and MAp44 levels did not differ between patients and controls. MASP-1 and MASP-2 levels correlated with HbA1c, and MASP levels decreased when glycaemic control improved. Since MASP-1 and MASP-2 have been shown to directly interact with blood coagulation, elevated levels of these proteins may play a role in the enhanced thrombotic environment and consequent vascular complications in diabetes.

Introduction

There is increasing evidence that the complement system, which is a part of the innate immune system, plays an important role in diabetes and the development of diabetic vascular complications (please refer to the excellent reviews by Phieler *et al* [1] and Hertle *et al* [2]). Plasma concentrations of several proteins of the complement system are elevated in patients with type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM), including the central component C3 [3,4]. Furthermore, deposits of complement activation products have been found in tissues from diabetes patients [5,6]. Many studies have shown that high levels of circulating complement factors and increased complement activation are associated with vascular complications of diabetes such as cardiovascular disease and diabetic nephropathy.

We have an interest in the lectin pathway of the complement system, which may play a role in the development of these complications. Mannan-binding lectin (MBL) and ficolins are the pattern recognition molecules in the lectin pathway. Upon binding of MBL or ficolins to a target molecule, activation of the lectin pathway is mediated by the MBL-associated serine proteases (MASPs) MASP-1 and MASP-2. The role of MASP-3 is not yet fully understood.[7-9] Two MBL-associated proteins, MAp19 and MAp44, which are alternative splicing variants of the *MASP2* and *MASP1* genes, respectively, have no protease activity. MAp44 was reported to have a regulatory function by displacing MASPs from the MBL complex and inhibiting lectin pathway activation. [10, 11]

Of the proteins mentioned above, MBL and ficolins have so far been studied in diabetes. MBL levels are elevated in patients with T1DM [12] and T2DM [13]. Among

diabetes patients, MBL and H-ficolin levels were higher in patients with diabetic nephropathy than in patients with normal renal function [,14-16]. In a prospective study high MBL levels were associated with progression to end-stage renal disease [17].

This evidence for a role of MBL in diabetes and diabetic vascular complications inevitably leads to the question whether levels of MASPs are also altered in diabetes and may contribute to diabetes complications. In the lectin pathway, MBL acts through its associated serine proteases, and MASPs circulate in plasma to a major extent in complex with MBL. Yet MASP levels have never been measured in diabetes patients. To our knowledge there is only one study available in which MASP-2 levels were measured in patients with T2DM who suffered from myocardial infarction [18]. Patients were then followed for a median period of 2.1 years for further cardiovascular events. Patients who suffered from cardiovascular events during that period had significantly lower MASP-2 levels at admission, but the significant association was lost after adjustment for cardiovascular risk factors. We have shown earlier that levels of MASP-1 and MASP-2 are altered in patients with cardio- and cerebrovascular diseases [19].

The aim of our present study was to measure for the first time plasma levels of MASP-1, MASP-2, MASP-3, and MAp44 in patients with T1DM and investigate possible associations with glycaemic control.

Materials and Methods

Patients and control subjects

We included 30 children and 45 adults with T1DM, and 17 children and 31 adults were included as non-diabetic, age and sex matched controls. All diabetes patients and control subjects were recruited at the University of Leeds. Control subjects were recruited through advertisement in the same hospital and unit where the patients were recruited. Other than background retinopathy, there were no significant microvascular complications in patients with T1DM and none were on any treatment other than insulin. We aimed to improve glycaemic control by adjusting insulin doses and regular patient contact, and collect repeat blood samples during the adult patients' routine clinic follow- up, usually occuring in 3-4 months. Due to loss to follow-up or missed appointments, repeat blood samples were obtained from only 26 adult patients.

Blood sampling was performed mid-morning after a light breakfast. Citrated plasma was separated within 2 hours of collection and stored frozen in aliquots until analysis. All participants, and in the case of underage individuals also their parents, gave written informed consent in accordance with the Declaration of Helsinki. The study was approved by the local ethics committee.

Laboratory measurements

We measured levels of MASP-1, MASP-2, and MASP-3 in citrated plasma samples which had been stored frozen in aliquots at -80°C until analysis. MASP-1 was determined with a competition ELISA using a MASP-1 specific antibody as described earlier [20]. Plasma levels of MASP-2 and MASP-3 were measured with commercial ELISA kits (Hycult Biotech, The Netherlands). MAp44 was determined with a time-resolved immunofluorometric assay (TRIFMA) using a catching antibody and a

biotinylated detecting antibody in a sandwich-type assay as described previously [10]. Inter-assay coefficients of variance of all assays were below 15%. Routine parameters were determined in the routine diagnostic laboratories of the Leeds General Infirmary hospital.

Statistical analysis

Statistical analysis was performed with IBM SPSS Statistics, version 21. We used Kolmogorov-Smirnov and Shapiro-Wilk tests to check the data for normal distribution. Since most parameters did not follow the normal distribution in all groups, all data are displayed as median with interquartile range (25^{th} percentile; 75^{th} percentile). The appropriate parametric or non-parametric statistical tests were applied as indicated. Bivariate correlations of parameters were analysed using Pearson or Spearman correlation coefficients. Parameters were compared between two or multiple groups using the appropriate parametric (t-test or analysis of variance (ANOVA)) or non-parametric (Mann-Whitney or Kruskal-Wallis test) methods. Differences between paired samples were tested by Wilcoxon or Friedman test. The Chi square test was used to compare categorical parameters between groups. A *P*-value of less than 0.05 was considered statistically significant.

Results

Characterisation of diabetes patients and control subjects

Our study population comprised the following groups: 1) children with T1DM and healthy control subjects matched for age and sex, and 2) adults with T1DM and healthy control subjects matched for age and sex. Demographic and clinical characteristics of these groups are shown in Tables 1 and 2.

Plasma levels of MASPs and MAp44 in diabetes patients and controls

MASP-1 and MASP-2 levels were significantly higher in children (Table 1) and adults (Table 2) with T1DM than in their respective control groups, whereas MASP-3 and MAp44 levels did not differ between patients and controls. When we compared MASP levels between all groups, MASP-1 levels were lowest in non-diabetic children and highest in adults with T1DM (Figure 1a). MASP-2 levels were lowest in non-diabetic children and young adults, and highest in adults with T1DM (Figure 1b). MASP-3 levels did not follow this trend and were lower in adults than in children irrespective of T1DM (Figure 1c).

Effect of glycaemic control on levels of MASPs and MAp44

There were significant correlations between MASP-1 levels and HbA1c in children with T1DM (Spearman correlation coefficient 0.456 (p=.011) and in adults with T1DM (0.482, p=<.001). In adults with T1DM, there was also a correlation between MASP-2 levels and HbA1c (0.437, p=<.001), and both MASP-1 and MASP-2 levels correlated with the duration of T1DM (MASP-1 0.346, p=.004; MASP-2 0.359, p=.003). Neither MASP-3 nor MAp44 levels did correlate with HbA1c in any patient group.

In a subgroup (n=26) of adult patients with T1DM, we measured levels of MASPs and MAp44 at baseline and 16±3 weeks after improving glycaemic control (shown in Table 3). Overall, a moderate but statistically significant reduction in HbA1c was not associated with significant reductions in MASP levels. However, patients whose HbA1c improved by at least 10% of the baseline value did show significant intraindividual reductions in MASP-1 and MASP-3. There was also a trend towards a reduction in MASP-2 levels, but the large variation in MASP-2 levels may be the reason for a non-significant result.

Taken together, these results suggest that blood glucose levels may represent a determinant of MASP levels, and in particular MASP-1 levels, in T1DM.

Discussion

Although MBL is well known to be elevated in diabetes patients and thought to be involved in vascular complications of diabetes, plasma levels of the MBL-associated serine proteases, MASP-1, MASP-2, and MASP-3, and the regulator MAp44 have not yet been studied in patients with diabetes. Here we show for the first time that MASP-1 and MASP-2 are elevated in patients with T1DM, that MASP-1 and MASP-2 levels correlate with HbA1c, and that glycaemic control may modulate MASP levels.

Our results suggest that levels of MASPs, in particular MASP-1 and MASP-2, may be linked to blood glucose levels. Recent data on MBL obtained in mouse models support this conclusion. MBL plasma levels were measured in mice before and seven weeks after inducing diabetes by streptozotocin. Diabetes induction led to an increase in MBL-C that was associated with the increasing plasma glucose levels. This study suggested that MBL levels increase in mice as a consequence of diabetes [21]. Other studies have shown that MBL knockout or insulin treatment protected hyperglycaemic mice from cardiac complications [22,23].

A possible link between hyperglycaemia and complement activation has been suggested by Fortpied *et al* [24]. They could show that MBL binds with high affinity to the glycation product fructoselysine and that this binding is associated with complement activation. Advanced glycation endproducts (AGEs) have been considered responsible for various adverse outcomes associated with insulin resistance and diabetes, such as inflammatory processes, endothelial damage, activation of blood coagulation, and vascular complications [25-27].

Based on the results of our present study we conclude that diabetes features not only elevate plasma levels of MBL but also its associated serine proteases. The increase of MASPs in diabetes may be induced by the same underlying mechanisms that are responsible for the increase of MBL as a consequence of hyperglycaemia, or MASP levels increase secondary to the increase of MBL as their binding protein. The circulating complexes of MBL and MASPs bind to AGEs and this induces conformational changes leading to activation of MASP-1 and consequently MASP-2. We and others have shown that both MASP-1 and MASP-2 directly interact with blood coagulation factors prothrombin, fibrinogen, factor XIII, and thrombin-activatable fibrinolysis inhibitor, and hence can promote fibrin formation (as recently summarised by us [9]). This could eventually result in thrombotic complications. We therefore hypothesise that the axis "hyperglycaemia $\rightarrow$ elevated levels of MBL, MASP-1, MASP-2 $\rightarrow$ binding to AGEs $\rightarrow$ activation of MASP-1 and MASP-2 $\rightarrow$ increased fibrin formation" may represent an important link between diabetes and its thrombotic vascular complications.

Limitations of our study include the case-control design and the relatively small sample size. As there were no data in the literature on levels of MASPs and MAp44 in patients with diabetes, our intention was to perform this as a pilot study. The data presented in this work pave the way for future larger, prospective as well as mechanistic studies to determine the role of MASP levels and lectin pathway activation in the development of diabetes and its vascular complications, with the potential to discover new therapeutic targets.

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LJ and ST performed the laboratory measurements and analysed the data. RA and RK recruited and characterised the patients and controls, and analysed the data. VS designed the study, analysed the data and wrote the manuscript. All authors revised the manuscript. This study was funded by the OPO Foundation (Zurich, Switzerland) (grant awarded to VS).

Conflict of Interest

The authors have no financial or commercial conflicts of interest to declare.

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Tables

Table 1. Characteristics and plasma levels of MASPs in children with T1DM and matched controls.

	T1DM children n=30	Controls n=17	*P*-value [a)]
Age (years)	14.9 (12.7; 15.6)	14.4 (11.9; 15.4)	.319
Weight (kg)	58.7 (52.4; 63.5)	50.8 (39.9; 60.2)	.068
BMI (kg/m^2)	21.9 (20.6; 22.9)	n.d.	-
Total cholesterol (mmol/l)	4.6 (4.0; 5.2)	n.d.	-
Diabetes duration (months)	73.1 (34.2; 104.2)	n.a.	-
HbA1c (%)	9.3 (8.1; 10.1)	n.d.	-
MASP-1 (µg/ml)	11.1 (9.3; 13.7)	7.9 (6.2; 11.5)	.007
MASP-2 (ng/ml)	420.9 (325.5; 509.8)	278.4 (201.3; 423.2)	.008
MASP-3 (µg/ml)	8.2 (7.0; 9.3)	7.6 (7.1; 9.2)	.364
MAp44 (µg/ml)	1.9 (1.5; 2.1)	1.8 (1.4; 1.9)	.298

Continuous data are shown as median (25th percentile; 75th percentile). n = number; n.d. = not determined; n.a. = not applicable; a) Mann-Whitney test.

Table 2. Characteristics and plasma levels of MASPs in adults with T1DM and matched controls.

	T1DM adults n=45	**Controls n=31**	***P*-value**
Age (years)	22.0 (19.0; 26.0)	23.0 (22.0; 26.0)	.390 [a)]
Weight (kg)	71.9 (64.7; 78.8)	71.0 (63.0; 80.0)	.747 [a)]
BMI (kg/m^2)	23.2 (20.8; 25.7)	23.2 (21.7; 25.0)	.865 [a)]
Smoking, yes:no (%)	8:36 (18:82)	1:30 (3:97)	.102 [b)]
Microvascular complications, yes:no (%)	10:31 (24:76)	0:31 (0:100)	.004 [b)]
Creatinine (µmol/l)	89.5 (82.0; 100.0)	89.0 (79.0; 95.0)	.273 [a)]
Total cholesterol (mmol/l)	4.4 (3.9; 5.2)	4.1 (3.7; 5.0)	.142 [a)]
Diabetes duration (months)	108.0 (55.5; 162.0))	n.a.	-
HbA1c (%)	9.0 (8.0; 9.9)	5.4 (5.1; 5.5)	<.001 [a)]
MASP-1 (µg/ml)	12.5 (10.6; 15.5)	9.7 (8.3; 13.6)	.003 [a)]
MASP-2 (ng/ml)	400.1 (330.7; 580.8)	290.4 (198.3; 390.1)	.001 [a)]
MASP-3 (µg/ml)	4.8 (4.2; 5.6)	5.6 (4.4; 6.7)	.058 [a)]
MAp44 (µg/ml)	1.7 (1.5; 1.9)	1.6 (1.5; 1.8)	.725 [a)]

Continuous data are shown as median (25th percentile; 75th percentile). n = number; n.d. = not determined; n.a. = not applicable; a) Mann-Whitney test; b) Chi square test.

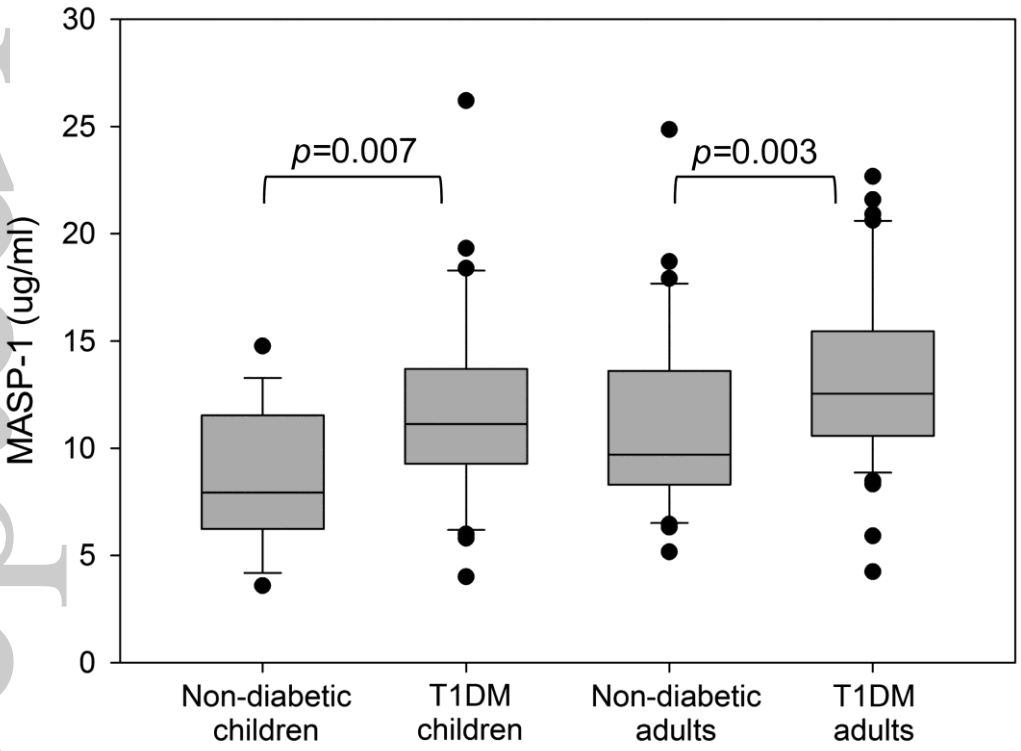
Table 3. Effect of glycaemic control on plasma levels of MASPs in adults with T1DM.

	Baseline	After improvement of glycaemic control	*P*-value [a)] for intraindividual change
All patients in this subgroup (n=26)			
HbA1c (%)	9.8 (9.2; 11.1)	9.0 (7.9; 10.5)	.001
MASP-1 (µg/ml)	16.5 (14.8; 20.2)	15.4 (13.4; 18.7)	.078
MASP-2 (ng/ml)	452.9 (315.7; 575.5)	362.6 (237.3; 537.0)	.170
MASP-3 (µg/ml)	4.7 (4.1; 5.7)	4.6 (3.9; 5.5)	.069
MAp44 (µg/ml)	2.0 (1.7; 2.2)	1.8 (1.6; 2.1)	.054
Patients whose glycaemic control improved most (HbA1c down by at least 10%) (n=12)			
HbA1c (%)	10.4 (9.3; 14.1)	8.2 (7.1; 9.7)	.002
MASP-1 (µg/ml)	16.9 (15.2; 21.5)	14.9 (12.8; 18.5)	.012
MASP-2 (ng/ml)	487.1 (350.0; 649.7)	441.5 (316.7; 635.9)	.136
MASP-3 (µg/ml)	5.3 (4.1; 6.5)	4.8 (3.8; 5.4)	.011
MAp44 (µg/ml)	1.9 (1.7; 2.2)	1.8 (1.7; 2.3)	.625

Data are shown as median (25th percentile; 75th percentile). n = number; a) Wilcoxon signed-rank test.

Figure legends

Fig. 1. Plasma levels of MASPs in patients with diabetes and control subjects showing (a) MASP-1, (b) MASP-2, and (c) MASP-3 levels. Boxes represent median, 25th and 75th percentiles, whiskers show the 10th and 90th percentiles, and dots are values outside these percentiles. Groups were compared using the Mann-Whitney test and the *p*-value is indicated; *ns* is non significant.



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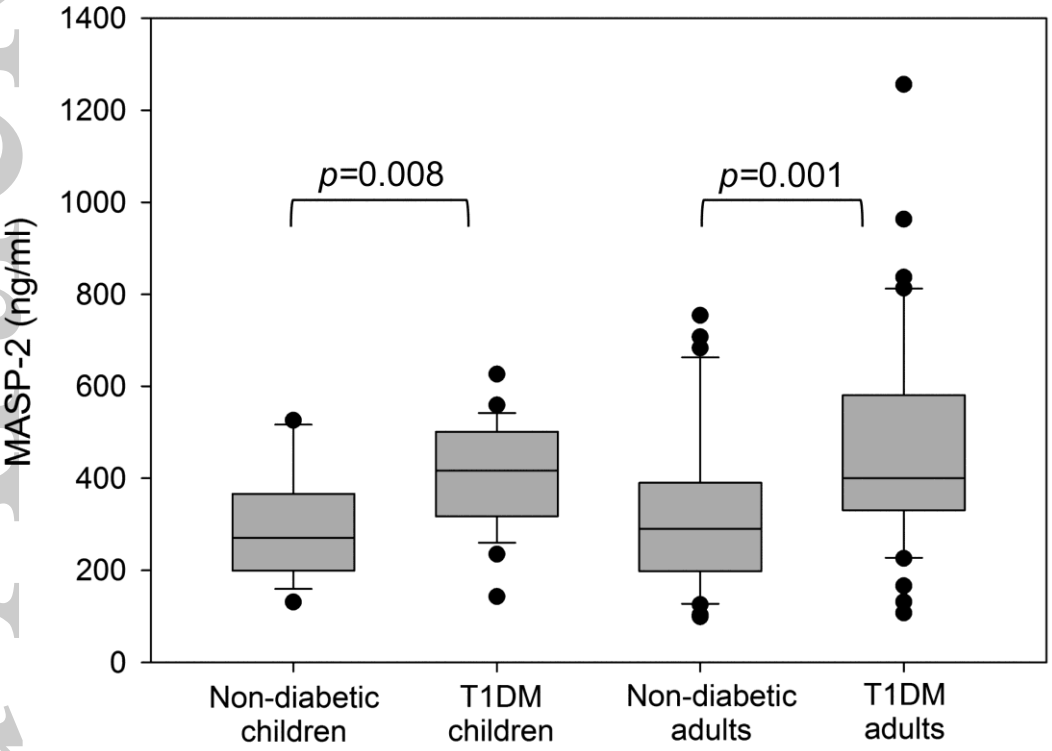

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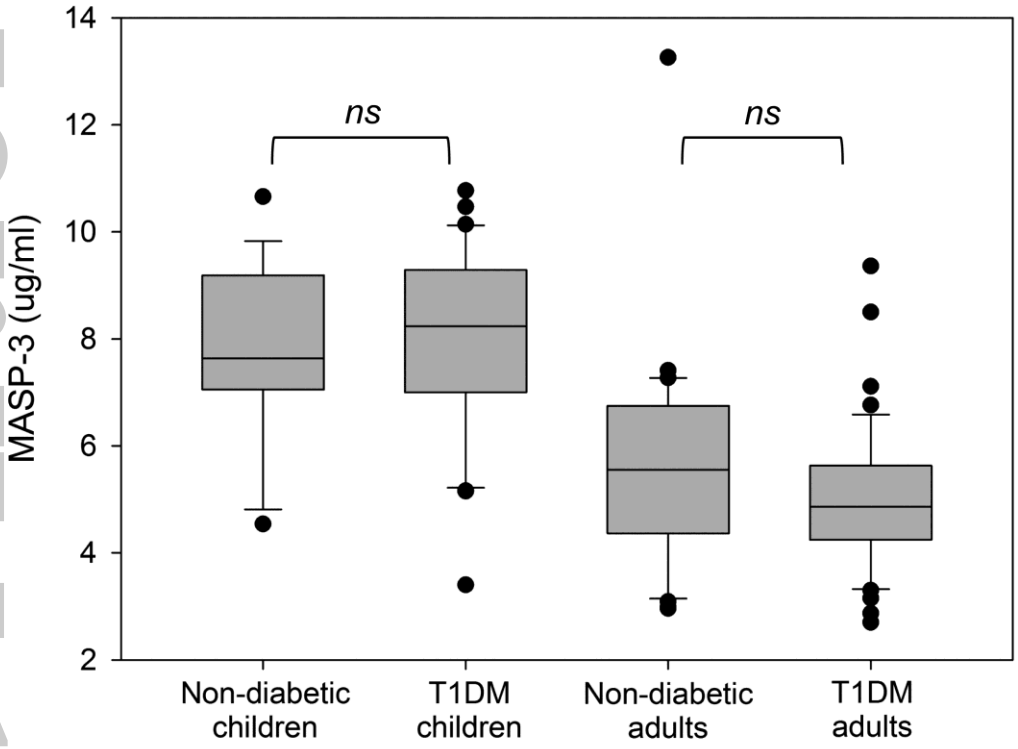

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